2010 Annual Report

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Organization: Colorado State University
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Award Title: Metabolic Engineering of Plants to Produce Precursors (Phloroglucinol and 1,2,4-butanetriol) of Energetic Materials

A. Scientific and Technical Objectives

Current chemical-based synthesis of energetic materials uses toxic ingredients and produces many environmentally undesirable wastes. In addition, chemical-based production is not sustainable because many of the chemicals used in making these precursors are petroleum-based. An alternative strategy is to produce precursors of energetic materials using a bio-based approach. Recently, significant progress has been made in identifying the genes and enzymes in microbes that can produce precursors of energetic materials. This knowledge can now be exploited to use plants for producing precursors of energetic materials in a sustainable manner. Plants offer many advantages in producing energetic ingredients. These include sustainability as they are renewable sources, likely to be highly cost-effective as they use light energy and CO_2 to produce sugars and other organic compounds that can be converted into desirable carbon compounds. The focus of our project is to use bacterial enzymes to engineer plant metabolism to produce precursors of energetic materials. The specific objectives of this proposal are: 1) Metabolic engineering of plants to produce high levels of phloroglucinol by introducing a bacterial gene that converts malonyl Co-A to phloroglucinol into plants. 2) Metabolic engineering of plants to produce butanetriol. This will be accomplished by introducing bacterial genes involved in butanetriol synthesis from xylose and arabinose. 3) To develop regeneration and robust stable transformation technologies for Miscanthus to introduce and express genes involved in synthesis of energetic materials.

B. Approach

Our approach is to express bacterial genes in plants to produce phloroglucinol and 1,2,4-butanetriol. Most plants naturally produce the precursors of these chemicals but not the enzymes to convert them to the precursors of energetic materials. However, bacteria have the enzymes that convert these precursors into end products. We will clone these bacterial genes, transfer them into plant transformation vectors under constitutive promoters and generate transgenic lines. The genes to be introduced are: xylose dehydrogenase, xylonate dehydratase, arabinose dehydrogenase, arabinonante dehydratase, benzoylformate decarboxylase, dehydrogenase and *PhlD*. Because of many available resources for Arabidopsis, we are using this plant as a model system to test this concept. Once proven, these constructs will be introduced into Miscanthus, a non-food crop that is known to grow well on marginal soils with minimum or no inputs. Transformation of plants will be done using Agrobacterium-based binary vectors using floral-dip method (in case of Arabidopsis) and regeneration of plants in tissue culture after co-cultivation with Agrobacterium or after particle bombardment (in case of Miscanthus). Most of our initial work will be performed in Arabidopsis and the best gene constructs will be transferred to Miscanthus, a grass species.

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We will be using a sterile hybrid of Miscanthus for these studies. Propagation of this variety is done through rhizomes.

C. Concise Accomplishments

We cloned and sequence verified and/or synthesized all genes needed for this work. These include *PhlD*, xylose dehydrogenase, xylonate dehydratase, arabinose dehydrogenase, L-arabinonate dehydratase and benzoylformate decarboxylase) that are necessary to produce these chemicals in plants. We then cloned all eleven genes (bacterial and synthetic) into plant transformation vectors, each with a different constitutive promoter. Arabidopsis transgenic lines were generated with *PhlD* for phloroglucinol production and xylose and arabinose pathway genes for the production of D- butanetriol and L-butanetriol, respectively. We obtained several independent transgenic plants expressing these constructs. Expression of all introduced genes was verified in T1 lines. We are in the process of obtaining homozygous lines of all these transgenic lines. Arabidopsis plants transformed with the *PhlD gene* are being analyzed for phloroglucinol production. Once we establish that we can produce these chemicals in Arabidopsis, these genes will be then introduced into *Miscanthus*, a non-food crop that is known to grow well on marginal soils with minimum or no inputs thus providing a renewable and less costly resource for the production of precursors of energetic material. Towards this goal, we are developing transformation methods for *Miscanthus*. We have done over three hundred hormonal combinations to optimize callus induction from Miscanthus organs. Of all the organs tested, roots from tissue culture grown plants and inflorescence yielded callus induction with 80-90% efficiency. We were able to regenerate whole plants form inflorescence calli with over 90% efficiency. We are now using the inflorescence calli to develop transformation methods for Miscanthus using Agrobacterium and gene gun.

D. Expanded Accomplishments

Objective 1: Metabolic engineering of plants to produce high levels of phloroglucinol by introducing a bacterial gene that converts malonyl Co-A to phloroglucinol into plants.

We cloned *PhlD* from *Pseudomonas fluorescens* and introduced into two entry vectors Impact vector 1.1 and pSAT4A. The entry vectors contain promoters and terminators for expression of the genes in plants. The promoter, gene, terminator cassette was digested from the entry vectors and cloned into binary vectors pRCS2-nptii and pBin. Both binary vectors were used to transform *Agrobacterium* and those transformants were then used to transform Arabidopsis plants. Seeds were collected from the transformed plants and were grown on kanamycin containing media. Transformants were obtained from the *PhlD* pRCS2-nptii construct (See Figure 1) and plants were transferred to soil (See Figure 2). Tissue from the leaves of transformants was analyzed for expression of the mRNA for *PhlD*. Expression was confirmed in different lines (Figure 3). These plants are selfed to generate homozygous lines. We recently obtained homozygous lines, which we are in the process of analyzing for the presence of phloroglucinol. In addition, we have synthesized *PhlD* gene to optimize for codon usage in Arabidopsis and generated transgenic lines with synthetic *PhlD*. Several lines expressing synthetic *PhlD* are identified by RT-PCR (see Figure 4).

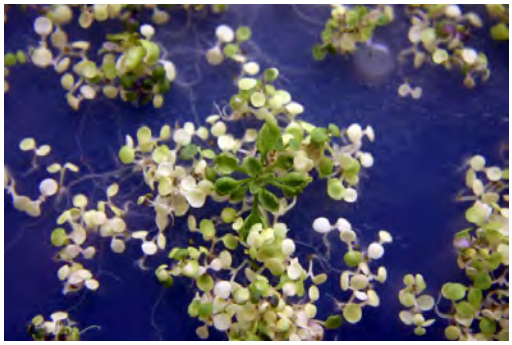

Figure 1. Left: Selection of transformants from PhlD transformation. **Right:** Transformants obtained with PhlD driven by 35S promoter are grown on kanamycin plates.

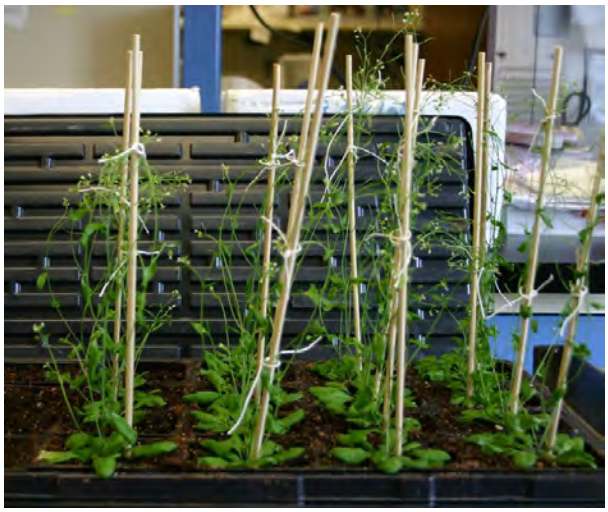

Figure 2. Transgenic Arabidopsis lines carrying PhlD gene driven by 35S promoter.

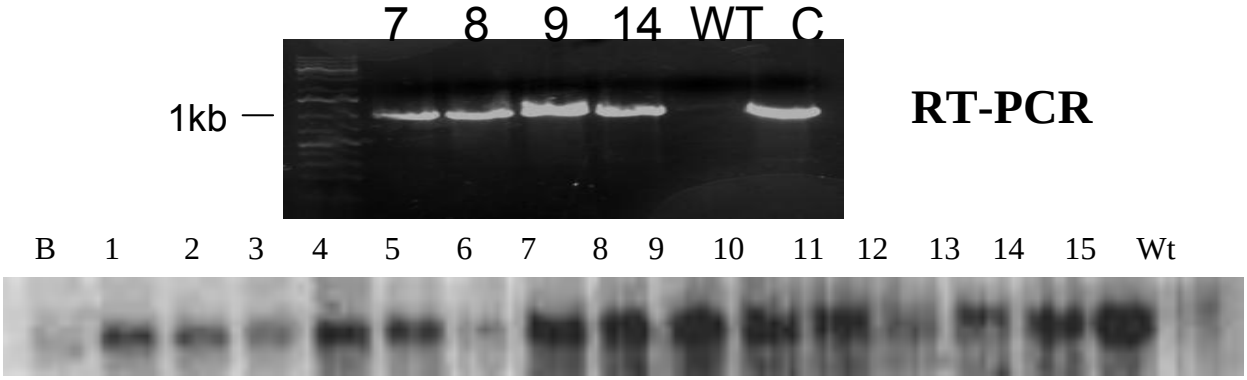

Figure 3. Expression of *PhlD* in transgenic lines. **Top:** RT-PCR analysis showing the levels of *PhlD* transcript in four transgenic lines and wild type (WT). C, positive control. **Bottom:** Northern blot. Numbers 1 to 15 are transgenic lines in which *PhlD* is driven by 35S promoter, B, transgenic line in which *PhlD* is driven by rubisco promoter. Wt, wild type.

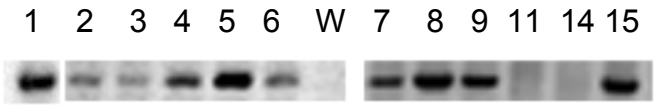

Figure 4. Expression of synthetic *PhlD* in transgenic lines. RT-PCR analysis showing the levels of *PhlD* transcript in twelve transgenic lines (indicated with numbers) and wild type (W).

Objective 2: Metabolic engineering of plants to produce butanetriol. This will be accomplished by introducing bacterial genes involved in butanetriol synthesis from xylose and arabinose.

Xylose dehydrogenase (XDG) was cloned from *Calobacter crescentus* and cloned into pSAT3A, xylonate dehydratase (XDT) was cloned from *E. coli* and cloned into pSAT4A, benzoylformate decarboxylase (BFD) was cloned from *Pseudomonas putida* and cloned into pSAT6A. Arabinose dehydrogenase (AD) and arabinonate dehydratase (ADT) were constructed synthetically based on the genes from *Burkholderia vietnamiensis* G4 and *Pseudomonas fluorescens* and were cloned into pSAT3A and pSAT4A, respectively. Xylose dehydrogenase (XDG), XDT, and BFD were also synthesized. The synthetic genes have altered codons that are optimized for expression in Arabidopsis. The pSAT vectors have the promoters and terminators for expression of the genes in plants.

To construct the binary vectors with XDG XDT, BFD (bacterial or synthetic) and AD, ADT, BFD (synthetic genes) each gene was cloned sequentially into a promoter vector and then moved the promoter with the gene into a plant transformation vector and verified by PCR. Verification of all constructs in plant transforation vectors is shown in Figures 5 to 7. We have transformed plants with all three constructs. T_o seeds for xylose pathway with bacterial genes are obtained. For xylose pathway with synthetic genes as well as arabinose pathway with synthetic genes, transgenic lines were obtained and expression of introduced genes at the mRNA level was verified using RT-PCR (see Figures 8 and 9)

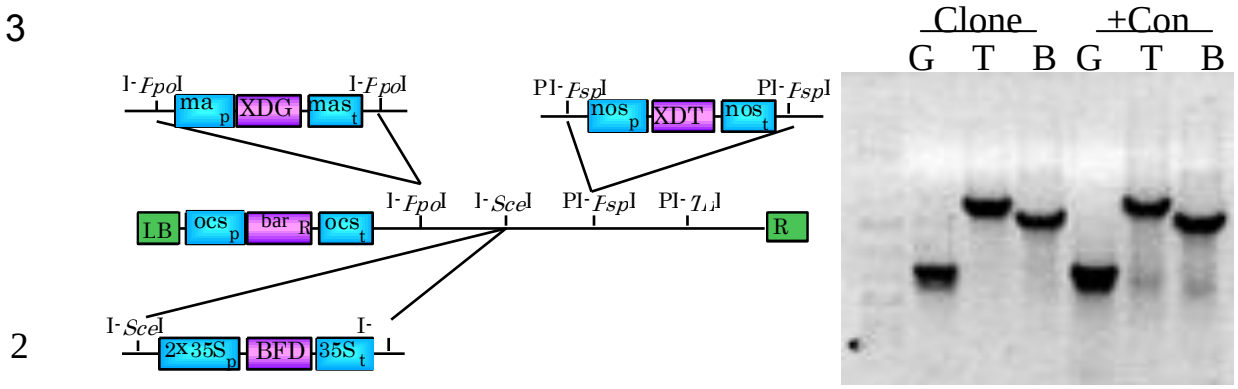

Figure 5. Left: Schematic diagram showing cloning of three bacterial genes of xylose pathway into a binary vector. 1. Inserted XDT into pSAT4A. Transferred cassette to pRCS2-ocs-bar; 2. Inserted BFD into pSAT6A. Transferred cassette to pRCS2-ocs-bar/XDT; 3. Inserted XDG into pSAT3A. Transferred to XDT/BFD/pRCS2-ocs-bar. **Right:** PCR verification of XDG, XDT and BFD in pRCS2-ocs-bar (Clone); +Cont, PCR from plasmids; G- XDG, T- XDT, B- BFD,

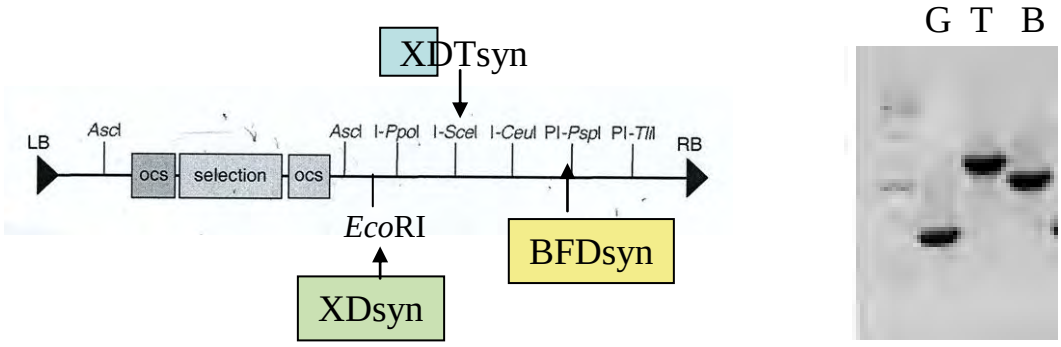

Figure 6. Left: Schematic diagram showing cloning of synthetic genes of xylose pathway into a binary vector. 1. Inserted XDT into pSAT4A. Transferred cassette to pRCS2-ocs-bar; 2. Inserted BFD into pSAT6A. Transferred cassette to pRCS2-ocs-bar/XDT; 3. Inserted XDG into pSAT3A. Transferred to XDT/BFD/pRCS2-ocs-bar. **Right:** PCR verification of XDG, XDT and BFD in pRCS2-ocs-bar (Clone).

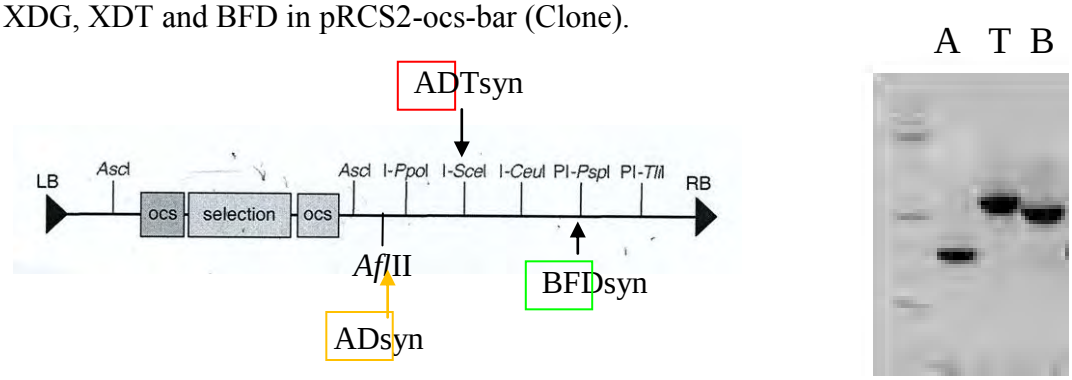

Figure 7. Left: Schematic diagram showing cloning of synthetic genes of arabinose pathway into a binary vector. Arabinose dehydrogenase (ADsyn), Arabinonate dehydratase (ADTsyn), Benzoylformate decarboxylase (BFDsyn) genes in a binary vector. **Right:** PCR verification of Arabinose dehydrogenase (A), Arabinonate dehydratase (T), Benzoylformate decarboxylase (B) in the construct.

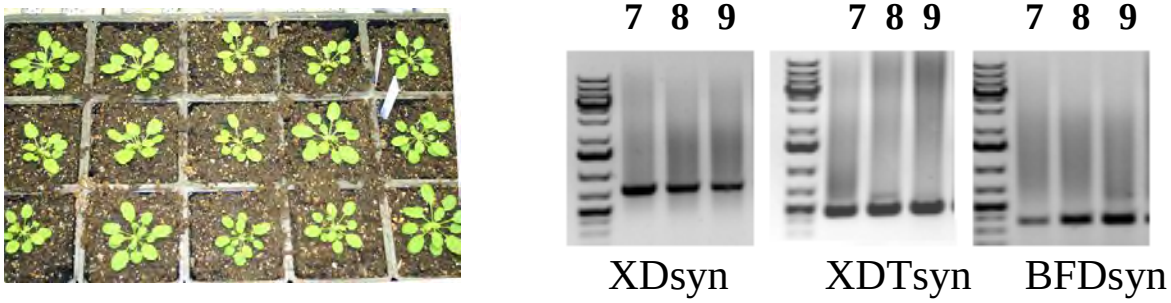

Figure 8. Left: Transgenic plants containing three synthetic genes of xylose pathway **Right:** RT- PCR verification of expression of all three genes using gene-specific primers in three plants (7, 8 and 9)

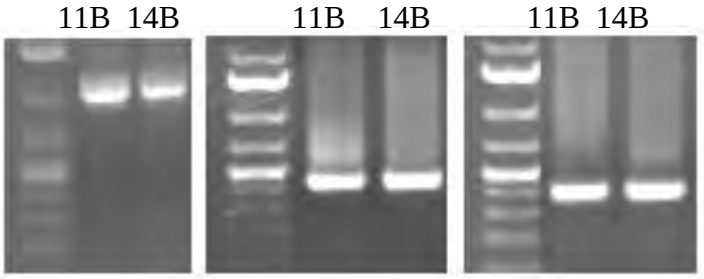

Figure 9. Left: Transgenic plants containing three synthetic genes of arabinose pathway **Right:** RT- PCR verification of expression of all three genes using gene-specific primers in two plants (11B, 14B).

Objective 3. To develop regeneration and robust stable transformation technologies for Miscanthus to introduce and express genes involved in synthesis of energetic materials.

Different plant parts (roots, leaves and inflorescence) from Miscanthus (*Miscanthus x giganteus*, a triploid species) were used for callus induction (Figure 10). We have tried 126 hormonal combinations for each tissue. Leaves did not produce callus in any hormonal combinations. Roots and inflorescence produced good calli with 80-90% frequency under a few hormonal combinations (Figure 11). We have developed methods for regeneration of plants from inflorescence callus with over 90% efficiency (see Figure 12)

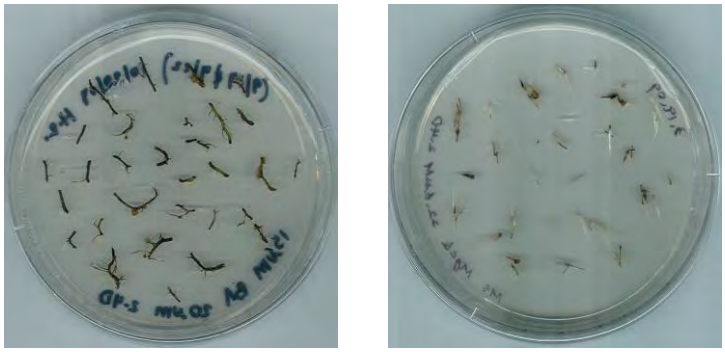

Figure 10. Induction of callus from leaves (left), roots (middle) and inflorescence (right)

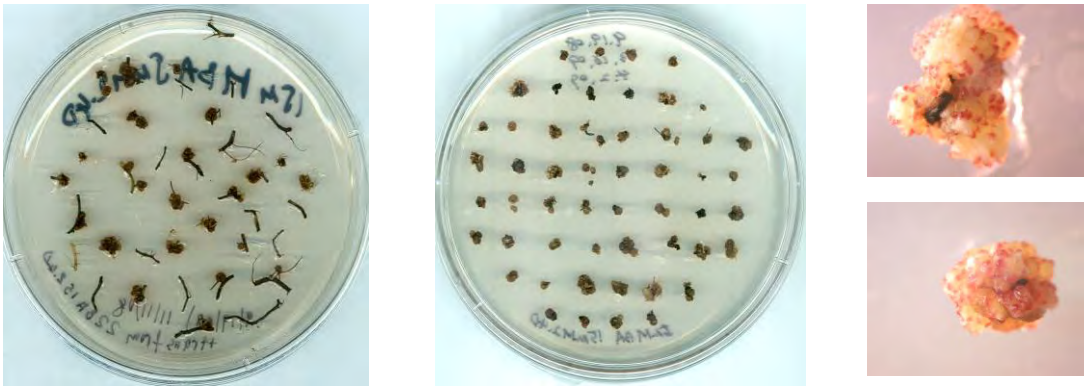

Figure 11. Calli induction from roots (left) and propagation of root calli (middle). Magnified view of two calli (right).

Figure 12. Regeneration of plants from callus derived from inflorescence. **From left to right:** 1) shoot induction, 2) close-up view of regenerating shoots, 3) root induction 4) regenerated plant.

We have constructed a fluorescent reporter fused nuclear protein (SR1) for transformation of Miscanthus (Fig. 13).

QuickTime™ and a
TIFF (Uncompressed) decompressor
are needed to see this picture.

YFP Light

Figure 13. Verification SR1-YFP construct. The fusion protein expression is verified in transgenic Arabidopsis plants (left, YFP). Bright field image is on the right (light).

E. Work Plan:

We will analyze bacterial and synthetic PhlD expression lines for the production of phloroglucinol. We will obtain homozygous lines for xylose pathway (for bacterial as well as synthetic genes) and arabinose pathway by screening T2 seeds. We will develop methods to detect and quantify D- and L-butanetriol from plant extracts. Homozygous lines expressing the xylose and arabinose pathway will be analyzed for the production of butanetriol. We will perform metabolomics analysis of transgenic lines of containing each construct. We will use the optimized regeneration conditions for transformation using fluorescent reporter systems. Transformation with different strains of Agrobacterium as well as direct gene transfer using gene gun method are being used. Once we establish robust transformation system we will then introduce all the constructs into Miscanthus.

F. Major Problems/Issues

No major problems were encountered. We were unable to clone the arabinose dehydrogenase and arabinonate dehydratase genes from the bacteria by PCR. We used these genes that are synthetically constructed, which are optimized for expression in Arabidopsis.

The cloning of the genes from the pSAT vectors into the pRCS2 vectors has proven to be a challenge. Due to the large size of the vectors, especially when they contained the cloned genes, cloning has been difficult. Different enzyme strategies have had to be developed and optimization of cloning has taken a longer time than expected. Furthermore, we also encountered problems with stability of large constructs in bacterial cells. Using different strains we solved this problem.

Callus induction from inflorescence took a while as it took 8 months for Miscanthus plants to flower. Because of this it has taken a long time to obtain calli from flowers.

G. Technology Transfer

No technology transfer as of now.

H. Foreign Collaborators and Supported Foreign National

Dr. Gul Shad Ali
Dr. Watanabe, Shizuoka University, Japan